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Short communication

Alleviation of proteolytic degradation of recombinant human bone morphogenetic protein-4 by repeated batch culture of Chinese hamster ovary cells

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ABSTRACT

The effects of culture temperature and pH on cell growth and recombinant human bone morphogenetic protein-4 (rhBMP-4) production were investigated to maximize rhBMP-4 production from Chinese hamster ovary cells. Cells were cultivated in a bioreactor at different culture pH levels (6.7, 6.9, 7.2, and 7.5) and temperatures (37 °C and 33 °C). The highest rhBMP-4 concentration (4.6 ± 0.5 μg/mL) was obtained at pH 7.2 and 37 °C. Regardless of culture temperature and pH, significant rhBMP-4 degradation via increased proteolytic activity was observed in the decline growth phase. Protease inhibitors such as EDTA and leupeptin were toxic and thus did not enhance rhBMP-4 production. High viability was achieved in repeated batch cultures with frequent medium exchanges. These cultures showed enhanced rhBMP-4 productivity, demonstrating the potential of repeated batch cultures as a means for improved rhBMP-4 production.

1. Introduction

Bone morphogenetic proteins (BMPs) are a group of cytokines involved in the regulation of bone organogenesis [1]. Recombinant human (rh) BMP-2 and rhBMP-7 have been used clinically to treat non-unions, long bone fractures, and spinal fusion [2]. A conserved multifunctional growth factor, rhBMP-4, was recently considered as a potential therapeutic agent for bone and cartilage repair and in the treatment of osteoarthritis and rheumatoid arthritis [1,3].

rhBMPs used in clinical applications are synthesized intracellularly as an inactive precursor in Chinese hamster ovary (CHO) cells. The precursor then undergoes proteolytic cleavage by proprotein convertases (PCs) within the secretory pathway to form functionally active dimers that are used in clinical applications. However, insufficient amounts of PCs in the secretory pathway result in decreased product yields [4–6]. Furthermore, rhBMPs such as rhBMP-2 and rhBMP-7 are prone to degradation in the decline growth phase during batch culture [5,6]. Thus, the maximum titer of rhBMPs in batch cultures of CHO cells is still below 10 μg/mL, though several strategies to improve product yield have been attempted [4–6].

Efficient methods to obtain high yields of high quality, biologically active rhBMP-4 must be developed to meet increasing demands. Despite the clinical importance of rhBMP-4, no studies have been conducted to optimize environmental factors for rhBMP-4 production in CHO cell cultures.

The aim of this study was to enhance rhBMP-4 production in CHO cell cultures. We investigated the effects of culture temperature and pH on rhBMP-4 production and proteolytic cleavage of rhBMP-4 to develop a strategy to increase the net rhBMP-4 yield by limiting proteolytic cleavage.

2. Materials and methods

2.1. Cell line and culture maintenance

A CHO cell line expressing rhBMP-4 (CHO-BMP-4) was established from CHO DG44 host cells using a dihydrofolate reductase/methotrexate (MTX)-mediated gene amplification system. The CHO-BMP-4 cell line was selected with a final concentration at 1 μM MTX (Sigma-Aldrich, St. Louis, MO, USA) and was adapted to grow in suspension cultures in a 125-mL Erlenmeyer flask on a Climo-Shaker (Adolf Kuhner AG, Birsfelden, Switzerland)

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at 110 rpm in a humidified 5% CO_2/air mixture at 37 °C. CD-OptiCHO (Invitrogen, Burlington, ON, USA) supplemented with 1 μM MTX and 8 mM glutamine was used for cultures.

2.2. Bioreactor culture

Exponentially growing cells were inoculated at 5.0×10^5 cells/mL into a 3-L BIOFLO 110 bioreactor (New Brunswick Scientific, Edison, NJ, USA) with a working volume of 1 L. The agitation speed was 50 rpm, and the dissolved oxygen concentration was controlled at 50% of air saturation. The culture temperatures were controlled at 33 °C and 37 °C. The culture pH was controlled at various pH levels (6.7, 6.9, 7.2, and 7.5) by manipulating CO_2 gas or adding 1 M $NaHCO_3$. Samples were collected daily from the bioreactor to determine the viable cell concentration using an automated cell counter (Countess, Invitrogen). Culture supernatants were aliquoted and stored at −70 °C for further analyses. Three independent cultures were generated at each temperature and pH level.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The secreted rhBMP-4 concentration in cell culture supernatants was quantified using a DuoSet BMP4 ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples were assayed in duplicate.

2.4. Western blot analysis

Western blot analysis was performed as described previously [7]. Briefly, an equal volume of the culture supernatants was loaded on 4–12% Bis-Tris NuPAGE gel with non-reducing conditions. The monoclonal antibody used for analysis was anti-human BMP-4 (R&D Systems).

2.5. Protease activity assay

Protease activity in the culture supernatants sampled from the bioreactor cultures was measured using the EnzChek protease assay (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, culture supernatants frozen at −70 °C were thawed and diluted in digestion buffer. Diluted samples were then incubated with boron-dipyrromethene fluorescent casein substrate at 24 ± 1 °C for 24 h. A sequential dilution of chymotrypsin (Sigma-Aldrich) was used as a positive control and an equivalent digestion buffer was used as a negative control.

2.6. Culture supernatant incubation

Exponentially growing cells were inoculated at 5.0×10^5 cells/mL in 125-mL Erlenmeyer flasks with a working volume of 50 mL on a Climo-Shaker at 110 rpm in a humidified 5% CO_2/air mixture at 37 °C. Approximately 15 mL of culture medium was sampled from the flasks at indicated time points. Culture supernatants, after centrifugation at 12,000*g* for 5 min to pellet cells, were transferred to 125-mL Erlenmeyer flasks and incubated on a Climo-Shaker at the same condition. Samples were collected daily from the flasks and stored at −70 °C for further analyses. Culture supernatants incubation experiments were performed three separate times.

2.7. Protease inhibitor screening

One milliliter aliquots of culture supernatants collected on day 5 from the shake flask cultures were transferred into 1.5-mL tubes. EDTA, aprotinin, leupeptin, or pepstatin A (all purchased from Sigma-Aldrich) were added to the culture supernatants at a final concentration of 1 mM, 8 μM, 0.2 mM, and 1 μM, respectively. Supernatants were then incubated on a Climo-Shaker in a humidified 5% CO_2/air mixture at 37 °C. Approximately 0.3 mL of culture supernatants were removed after 24, 72, and 120 h incubation and were frozen at −70 °C to determine rhBMP-4 concentrations. The experiments were replicated three times.

2.8. Repeated batch cultures

Exponentially growing cells were inoculated at 5.0×10^5 cells/mL in 125 mL-Erlenmeyer flasks with a working volume of 30 mL and incubated on a Climo-Shaker in a humidified 5% CO_2/air mixture at 37 °C. Two different sets of repeated batch cultures were carried out. Cells in the late exponential growth phase were passaged at various dilutions. For the first set, 90% of culture medium was removed from the flasks and replaced with an equivalent volume of fresh culture medium every 3 days. For the second set, 60% of culture medium was exchanged with fresh culture medium daily from day 3 to day 9 and from day 17 to day 21. From day 10 to day 16, 30% of culture medium was exchanged with fresh culture medium every 12 h. Cultures were performed three separate times.

2.9. Statistical analysis

Reported values are shown as mean ± standard deviation, $n \geq 3$. The statistical significance of differences was defined by $P < 0.05$ according to the two-tailed Student's *t*-test.

3. Result and discussion

We developed a CHO cell line expressing rhBMP-4 (CHO-BMP-4) to meet increasing demands for high-level production of rhBMP-4. CHO-BMP-4 cells were cultivated at two different temperatures (37 °C and 33 °C) in a bioreactor with pH control to determine the optimal culture environment for rhBMP-4 production. Culture pH was controlled at 6.7, 6.9, 7.2, and 7.5.

3.1. Effect of culture temperature and pH on cell growth and rhBMP-4 production

Culture temperature and pH considerably affected cell growth and rhBMP-4 production (Fig. 1). Lowering the culture temperature from 37 °C to 33 °C considerably suppressed cell growth while extending cell culture longevity at all pH levels (Fig. 1A and B). Cells were unable to proliferate at pH 6.7 at any temperature. The optimal culture pH for cell growth at 37 °C was 6.9–7.2, consistent with the optimal pH range (6.8–7.4) previously reported for other CHO cell lines [8–11]. The specific growth rate (μ) and maximum viable cell concentration (MVCC) obtained at pH 6.9–7.2 and 37 °C were significantly higher at pH 6.9–7.2 and 37 °C than at 33 °C according to a two-tailed Student's *t*-test ($P < 0.001$).

Lowering the culture temperature in CHO cell cultures is a well-established and useful means of enhancing the production of recombinant proteins, such as antibodies [8,12], flag-tagged COMP angiopoietin-1 [9], human growth hormone [13], interferon-β [14], and erythropoietin [15] because it promotes both prolonged culture longevity and elevated specific productivity (q). However, the effect of lowering the culture temperature on q is not generalized because many CHO cell lines did not show enhanced q at reduced temperature [16].

Lowering the culture temperature did not increase rhMBP-4 production, but rather decreased it (Fig. 1A and B). Lower temperatures did not increase specific rhBMP-4 productivity ($q_{rhBMP\text{-}4}$) of CHO-BMP-4 cells regardless of culture pH. Furthermore, rhBMP-4